IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re-application of
Pagniez et al.

Serial N° : 09/821,463
Filed : 06/12/2001

Group art unit : 1638
Examiner : Georgia L. Helmer

For : Method for obtaining transgenic plants expressing a protein with activity producing hydrogen peroxide by transformation by *Agrobacterium rhizogenes.*

DECLARATION UNDER §1.132

Hon. Commissioner of Patents and Trademarks

Sir:

I, Alain TOPPAN, residing at Domaine de Sandreau F-31700 MONDONVILLE (France)

Declare and say :

I. I am citizen of France.

II. I am graduated from the University Paul Sabatier (Toulouse, FRANCE) where I got a Doctorate of 3rd cycle in 1977 followed by a Doctorat d'Etat in Biological Sciences in 1983.

III. I am currently working as the Laboratory Head Manager in Biogemma laboratory located, Domaine de Sandreau, 31700 Mondonville (France).

IV. I am an inventor of the present application and I am aware that the Examiner alleged that the invention as claimed fails to comply with the enablement requirement. Particularly, I am aware that the Examiner alleged that the method according to the invention do not provide enablement for other plants or genes.

V. However, the invention as claimed has been clearly exemplified in the specification: examples 1.1 (Transformation of rape with the wheat germin gene and selection of the transformants by a colorimetric test on the root) and 2 (Use of the peroxidase-based selection method for obtaining transgenic plants (rape) expressing a second gene of interest : the gene encoding a protein with endochitinase activity) are complete working examples.

VI. The method as claimed has been used as a routine method in my laboratory, notably for transforming rape, cauliflower and tobacco.

VII. In addition to these examples, at the time the invention was filed, oilseed rape, cauliflower, chicory, sunflower, Jerusalem artichoke, tomato, sweet potato, radish, cucumber, tobacco and numerous other plant species were known to give hairy roots culture after transformation by *Agrobacterium rhizogenes* and protocols for selection and regeneration were also available for these plants, making it thus possible, in my opinion, to perform the method of the invention on these plants.

The general review "Use of Ri-mediated transformation for production of transgenic plants" cites in table 1 numerous plant species leading to transgenic plants using *Agrobacterium rhizogenes* mediated transformation. As indicated in table 1, the major part of the references is dated before the priority date of the claimed invention.
In my opinion, it is also clear from this review that there was no technical obstacle for transformation, even for the species which are indicated as transformed after the priority date.

VIII. According to the claimed invention, a gene encoding an H_2O_2 producing protein is used. The nature of the gene does not matter as long as it is a gene encoding an H_2O_2 producing protein, as the claimed method specifically contains a step of visually detecting H_2O_2 produced by this protein. Numerous examples of genes encoding H_2O_2 producing proteins are cited in the specification (page 4 lines 13-27). I can not scientifically foresee why use of each of these genes would not work in the claimed method.

IX. The undersigned Declarant declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true ; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 12th day of December 2005

Alain Toppan

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INVITED REVIEW:

USE OF RI-MEDIATED TRANSFORMATION FOR PRODUCTION OF TRANSGENIC PLANTS

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(Received 25 September 2000; accepted 30 March 2001; editor C. L. Armstrong)

SUMMARY

Agrobacterium rhizogenes-mediated transformation has been used to obtain transgenic plants in 89 different taxa, representing 79 species from 55 genera and 27 families. A diverse range of dicotyledonous plant families is represented, including one Gymnosperm family. In addition to the Ri plasmid, over half these plants have been transformed with foreign genes, including agronomically useful traits. Plants regenerated from hairy roots often show altered plant morphology such as dwarfing, increased rooting, altered flowering, wrinkled leaves and/or increased branching due to *rol* gene expression. These altered phenotypic features can have potential applications for plant improvement especially in the horticultural industry where such morphological alterations may be desirable. Use of *A. rhizogenes* and *rol* gene transformation has tremendous potential for genetic manipulation of plants and has been of particular benefit for improvement of ornamental and woody plants.

Key words: Agrobacterium rhizogenes; Ri phenotype; *rol* genes; hairy roots; altered phenotype.

INTRODUCTION

Agrobacterium rhizogenes is a soil bacterium responsible for the development of hairy root disease on a range of dicotyledonous plants (Tepfer, 1990). *In vitro*, hairy roots are easily distinguished by their rapid, highly branching growth on hormone-free media and plagiotropic root development (Tepfer, 1989; Fig. 1*a*). This phenotype is caused by genetic transformation in a manner similar to the development of crown gall disease by *A. tumefaciens*. Infection of wound sites by *A. rhizogenes* is followed by the transfer, integration and expression of T-DNA (TR-DNA and TL-DNA in agropine strains) from the Ri (root-inducing) plasmid and subsequent development of the hairy root phenotype (Grant et al., 1991). Hairy roots can be selected in a wide range of plant species, many of which can be regenerated into plants, often spontaneously (Table 1; Fig. 1*b*). Plants regenerated from hairy roots often exhibit an altered phenotype characterized by several morphological changes including wrinkled leaves, shortened internodes, reduced apical dominance, reduced fertility, altered flowering, and plagiotropic roots (Tepfer, 1989; Fig. 1*c*). The characteristic phenotypic changes are due predominately to the TL-DNA (Taylor et al., 1985). Insertional mutagenesis in the TL-DNA has identified four loci (*rol*A, B, C, D) involved in hairy root formation (White et al., 1985). Transformation of individual *rol* genes into plants has provided information on the function and phenotype induced by these genes, both individually and in combination. In tobacco, the combined expression of *rol*A, B and C loci confers the full hairy-root phenotype (Schmülling et al., 1988; Mariotti et al., 1989), whereas plants transgenic for single *rol* genes or various combinations show distinct specific growth abnormalities (Table 2).

A variety of dicotyledonous plants are susceptible to *A. rhizogenes*. Tepfer (1990) lists 116 dicotyledonous species for

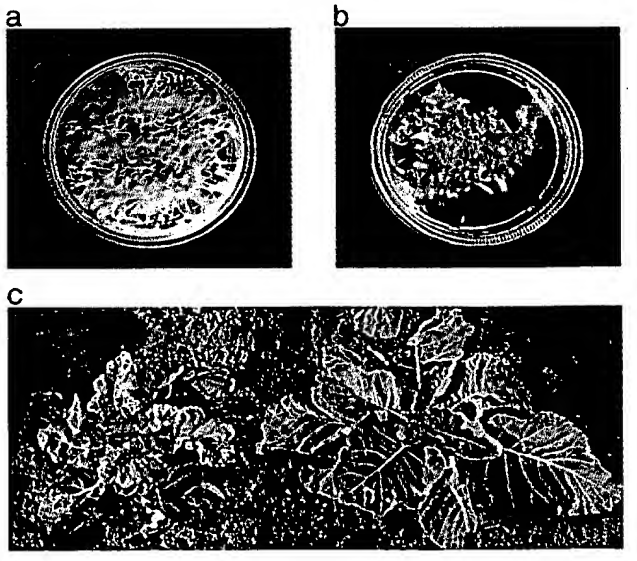

FIG. 1. Hairy roots and shoots of Basta-resistant Conds kale. *a*, Hairy root culture 3 wk after transfer to hormone-free medium. *b*, Shoot regeneration 3 wk after transfer to regeneration medium. *c*, Transgenic (*left*) and control (*right*) plants 8 wk after transplanting to the field, showing the effect of the Ri phenotype.

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TABLE 1

TRANSGENIC PLANTS OBTAINED FROM *AGROBACTERIUM RHIZOGENES*-MEDIATED TRANSFORMATION[a]

Family Genus and species	Common name	Gene(s) introduced[b]	Reference[c]
Actinidiaceae			
Actinidia deliciosa	Kiwifruit	WT	Yamakawa and Chen, 1996
Apiaceae (=Umbelliferae)			
Daucus carota	Carrot	WT	David et al., 1984
Foeniculum vulgare	Fennel	WT	Mugnier, 1988
Pimpinella anisum	Anise	WT	Andarwulan and Shetty, 1999
Apocynaceae			
Catharanthus roseus	Periwinkle	WT	Brillanceau et al., 1989
Vinca minor	Lesser periwinkle	NPTII, GUS	Tanaka et al., 1994
Araliaceae			
Panax ginseng	Ginseng	WT	Yang and Choi, 2000
Asteraceae (=Compositae)			
Artemisia annua	Sweet wormwood	WT	Banerjee et al., 1997
Cichorium intybus	Chicory	WT	Sun et al., 1991
Rudbeckia hirta	Black-eyed Susan	WT	Daimon and Mii, 1995
Brassicaceae (=Cruciferae)			
Arabidopsis thaliana	Mouse ear cress	WT	Pavingerova and Ondrej, 1986
Armoracia lapathifolia	Horseradish	WT	Noda et al., 1987
Brassica campestris (syn. *rapa*)			
var. *pekinensis*	Chinese cabbage	NPTII, EAS	Christey et al., 1999a
var. *rapifera*	Turnip	GUS, NPTII, ALS	Christey and Sinclair, 1992
B. napus			
var. *biennis*	Forage rape	GUS, NPTII, ALS	Christey and Sinclair, 1992
var. *oleifera*	Oilseed rape	NPTII	Boulter et al., 1990
	Rapid cycling	NPTII	Boulter et al., 1990
var. *rapifera*	Swede (rutabaga)	*bar*	Christey and Braun, 2001
B. oleracea			
var. *italica*	Broccoli	NPTII, EAS	Christey et al., 1999a
var. *gemmifera*	Brussels sprouts	NPTII	Christey et al., 1999a
var. *capitata*	Cabbage	NPTII, GUS	Christey et al., 1999a
var. *botrytis*	Cauliflower	NPTII, GUS	Christey et al., 1999a
var. *acephala*	Forage kale	GUS, NPTII, ALS	Christey and Sinclair, 1992
	Rapid cycling	NPTII, GUS	Christey et al., 1997
var. *acephala*	Ornamental kale	WT	Hosoki et al., 1989
Caricaceae			
Carica papaya	Papaya	NPTII, GUS	Cabrera-Ponce et al., 1996
Casuarinaceae			
Allocasuarina verticillata		WT	Phelep et al., 1991
Convolvulaceae			
Convolvulus arvensis	Morning glory	WT	Mugnier, 1988
Ipomoea batatas	Sweet potato	NPTII, GUS	Otani et al., 1993
I. trichocarpa	Blue morning glory	NPTII, GUS	Otani et al., 1996
Crassulaceae			
Kalanchoe daigremontiana	Devil's backbone	WT	White et al., 1985
Cucurbitaceae			
Cucumis sativus	Cucumber	NPTII	Trulson et al., 1986
Ebenaceae			
Diospyros kaki	Japanese persimmon	WT	Tao et al., 1994
Fabaceae (=Leguminosae)			
Anthyllis vulneraria	Kidney vetch	NPTII, *ipt*	Stiller et al., 1992
Astragalus sinicus	Chinese milk vetch	GUS	Cho et al., 1998
Crotalaria juncea	Sunn hemp	WT	Ohara et al., 2000
Glycine argyrea	Wild soybean	NPTII	Kumar et al., 1991
G. canescens	Wild soybean	NPTII	Rech et al., 1989
Lotus angustissimus		NPTII, GUS	Nenz et al., 1996
L. corniculatus	Bird's-foot trefoil	*Sn*	Damiani et al., 1999
L. japonicus		WT	Stiller et al., 1997
Medicago arborea		HPT	Damiani and Arcioni, 1991
M. sativa	Alfalfa/lucerne	WT	Golds et al., 1991
M. truncatula		NPTII	Thomas et al., 1992
Onobrychis viciifolia	Sainfoin	WT	Golds et al., 1991
Robinia pseudoacacia	Black locust	NPTII	Han et al., 1993
Stylosanthes humilis		NPTII	Manners and Way, 1989
Gentianaceae			
Eustoma grandiflorum	Lisianthus	NPTII, GUS	Handa, 1996

TABLE 1. *continued*

Family Genus and species	Common name	Gene(s) introduced[b]	Reference[c]
Gentiana cruciata	Gentian	GUS	Momčilović et al., 1997
G. punctata		GUS	Vinterhalter et al., 1999
G. purpurea		WT	Momčilović et al., 1997
G. scabra		WT	Suginuma and Akihama, 1995
G. triflora×*G. scabra*		WT	Hosokawa et al., 1997
Geraniaceae			
Pelargonium fragrans	Nutmeg geranium	WT	Pellegrineschi and Davolio-Mariani, 1996
P. graveolens	Lemon geranium	WT	Pellegrineschi et al., 1994
P. odoratissimus	Apple geranium	WT	Pellegrineschi and Davolio-Mariani, 1996
P. quercifolia	Oak-leaved geranium	WT	Pellegrineschi and Davolio-Mariani, 1996
Labiatae			
Ajuga reptans		GUS	Uozumi et al., 1996
Linaceae			
Linum usitatissimum	Flax	WT	Zhan et al., 1988
Myrtaceae			
Verticordia grandis		NPTII, GUS	Stummer et al., 1995
Papaveraceae			
Papaver somniferum	Opium poppy	WT	Yoshimatsu and Shimomura, 1992
Pinaceae			
Larix decidua	European larch	NPTII, *aro*A, BT	Shin et al., 1994
Primulaceae			
Anagallis arvensis	Pimpernel	WT	Mugnier, 1988
Rosaceae			
Malus pumila	Apple	WT	Lambert and Tepfer, 1992
Prunus avium×*P. pseudocerasus*	Cherry rootstock	WT	Gutièrrez-Pesce et al., 1998
Rosa hybrida	Hybrid tea rose	NPTII, GUS	Firoozabady et al., 1994
Rutaceae			
Citrus aurantifolia	Mexican lime	NPTII, GUS	Pérez-Molphe-Balch and Ochoa-Alejo, 1998
Salicaceae			
Populus tremula	Aspen	NPTII, GUS	Tzfira et al., 1996
P. trichocarpa×*P. deltoides*	Cottonwood	WT	Han et al., 1997
Scrophulariaceae			
Antirrhinum majus	Snapdragon	*bar*, NPTII	Hoshino et al., 1998
Digitalis lanata	Grecian foxglove	WT	Pradel et al., 1997
Scoparia dulcis		*bar*	Yamazaki et al., 1996
Solanaceae			
Atropa belladonna	Deadly nightshade	*bar*	Saito et al., 1992
Datura arborea		WT	Giovannini et al., 1997
D. sanguinea		WT	Giovannini et al., 1997
Hyoscyamus muticus	Egyptian henbane	WT	Oksman-Caldentey et al., 1991
Lycopersicon esculentum	Tomato	NPTII	Shahin et al., 1986
L. peruvianum		NPTII	Morgan et al., 1987
Nicotiana debneyi		NPTII	Davey et al., 1987
N. glauca	Tree tobacco	WT	Sinkar et al., 1988
N. hesperis		WT	Walton and Belshaw, 1988
N. plumbaginifolia		NPTII	Davey et al., 1987
N. tabacum	Tobacco	NPTII	Hatamoto et al., 1990
Nierembergia scoparia	Tall cupflower	WT	Godo et al., 1997
Petunia hybrida	Petunia	WT	Ondrej and Biskova, 1986
Solanum dulcamara	Nightshade	NPTII, *rol*	McInnes et al., 1991
S. nigrum	Black nightshade	NPTII	Davey et al., 1987
S. tuberosum	Potato	NPTII, GUS	Visser et al., 1989
Vitaceae			
Vitis vinifera	Grapevine	NPTII, GUS	Nakano et al., 1994

[a] Updated from Christey (1997).

[b] WT, a wild-type *A. rhizogenes* strain was used. Otherwise the gene(s) introduced are listed: ALS, mutant acetolactate synthase; *aro*A, mutant 5-enolpyruvylshikimate-3-phosphate synthase; *bar*, phosphinothricin acetyltransferase; BT, *Bacillus thuringiensis* toxin; EAS, antisense version of ACC oxidase; GUS, β-glucuronidase; HPT, hygromycin phosphotransferase; *ipt*, isopentenyl transferase; NPTII, neomycin phosphotransferase II; *rol*, root loci genes; *Sn*, maize anthocyanin regulatory gene.

[c] This is not a comprehensive list, but provides one key reference for each plant.

which stable, transformed hairy root cultures have been reported, with plants regenerated from 37 species. In 1997, Christey reported transgenic plants had been regenerated from hairy roots of 60 different taxa, representing 51 species from 41 genera and 23 families including one Gymnosperm family, Pinaceae (Christey, 1997). A recent literature survey now indicates that transgenic plants have been obtained after *A. rhizogenes*-mediated transformation in 89 different taxa, representing 79 species from 55 genera

and 27 families (Table 1). The three new families now represented are Araliaceae, Caricaceae and Rutaceae due to the regeneration from hairy roots of ginseng, papaya and Mexican lime (Table 1). In addition to the Ri plasmid, over half these plants have been transformed with foreign genes. A diverse range of dicotyledonous plant families are represented, including one Gymnosperm family, with 14 or more examples from each of the Fabaceae, Brassicaceae and Solanaceae families. There are still no examples of transgenic monocotyledonous plants, but onion (Dommisse et al., 1990) and asparagus (Hernalsteens et al., 1993) have been reported as hosts for *A. rhizogenes*. In addition, transient β-glucuronidase (GUS) expression has been demonstrated in wheat cells using *A. rhizogenes* (Uzé et al., 2000).

A. rhizogenes-derived hairy roots and plants have application for many areas of research. For example, hairy root cultures have been used extensively in root nodule research (Díaz et al., 1989; Quandt et al., 1993), for artificial seed production (reviewed in Uozumi and Kobayashi, 1997), for production of plant secondary metabolites (reviewed in Hamill and Lidgett, 1997), as an experimental system to study responses to chemicals (Downs et al., 1994; Mugnier, 1997), and to study interactions with other organisms such as nematodes (Kifle et al., 1999), mycorrhizal fungi and root pathogens (reviewed in Mugnier, 1997). Root cultures established by *A. rhizogenes*-mediated transformation are widely used as a source of useful compounds due to their rapid growth in hormone-free medium and the relatively high production of secondary metabolites compared with the starting plant material. In addition, over recent years there has been increased interest in the use of *A. rhizogenes* due to the effect of *rol* genes on plant morphology and development and the ability to introduce foreign genes via *A. rhizogenes*-mediated transformation. In this review, use of *A. rhizogenes* is confined to specific uses with potential applications for plant breeding and plant improvement including: root system alteration, use of *A. rhizogenes* and *rol* genes for altered phenotype, and the introduction of desirable foreign genes. In addition, an overview is presented of the current status of knowledge concerning *rol* gene function.

Rol Gene Function

A. rhizogenes Ri plasmids have been assigned to three classes: mannopine, agropine or cucumopine, depending on the opine synthesized and degraded. In mannopine and cucumopine type plasmids the T-DNA consists of a single region, whereas in agropine types, such as strain A4, two T-DNA regions are present on the Ri plasmid for transfer into plant cells. These two T-DNA regions can be integrated separately into the plant genome. The TL-DNA contains 18 ORFs including the *rol*A, *rol*B, *rol*C, and *rol*D genes, which correspond to ORFs 10, 11, 12, and 15. The TR-DNA contains genes (*aux*1, *aux*2, *rol*B TR, *mas*1, *mas*2, and *ags*) for the synthesis of agropine and the biosynthesis of auxin (reviewed in Grant et al., 1991; Lambert and Tepfer, 1992; van der Salm et al., 1996).

The *rol* genes are responsible for the observed phenotype of plants regenerated after Ri-mediated transformation. However, the exact mechanism whereby the *rol* genes cause this phenotype is not fully known. In addition to the visible phenotypic changes, alterations in levels of and sensitivity to several hormones have been noted in a range of plants.

The function of the rolA protein is unknown as its sequence shares no homology with other polypeptides (Levesque et al., 1988). However, promoter GUS fusion studies indicate that it has a constitutive promoter with expression mainly in stems (Hänisch ten Cate et al., 1988). Promoter studies indicate that the upstream region has a domain similar to the auxin-regulated gene in soybean, which was involved in auxin induction (McClure et al., 1989), suggesting auxin may regulate *rol*A expression.

Filippini et al. (1996) have shown that rolB protein over-expressed in *E. coli* has tyrosine phosphatase activity and that in transformed carrot cells it is localized in the plasma membrane. These results suggest that a kinase/phosphatase cascade plays a major role in the signal transduction of auxin. The *rol*B gene has also been shown to encode for a β-glucosidase which hydrolyzes indole-β-glucosides, suggesting a role in the release of active auxins from inactive β-glucosides (Estruch et al., 1991a). However, it is unclear if this is functional *in vivo* as an increase in indole-3-acetic acid (IAA) has not been reported in *rol*B transformed plants. *Rol*B introduced into a range of plants causes an auxin-like activity with enhanced adventitious root formation (Table 2). Shen et al. (1990) demonstrated an increased sensitivity to auxin of transformed roots in experiments involving short-, medium-, and long-term effects.

*Rol*C has a cytokinin-like action as the phenotype changes induced such as diminished apical dominance, increased shoot number and reduced chlorophyll are characteristics associated with cytokinin action. The *rol*C gene encodes a cytokinin-β-glucosidase and is capable of hydrolyzing cytokinin-β-glucosidases when expressed in *E. coli*, increasing the level of free cytokinins (Estruch et al., 1991b). However, subsequent studies have shown it is unclear whether this enzyme operates *in vivo* as free cytokinin levels were not enhanced in transgenic 35S-*rol*C tobacco, nor were cytokinin conjugates decreased (Nilsson et al., 1993). Nilsson et al. (1996) studied in detail levels of free and conjugated cytokinins in 35S-*rol*C and rbcS-*rol*C transgenic aspen. Levels of isopentenyl adenosine (iPA) were decreased, while zeatin 9-ribosides (ZR) increased dramatically. However, this increase was not linked to a decrease in glucosidic conjugates as they also increased. Z-family cytokinins increased, suggesting increased synthesis of Z-family cytokinins which induces conjugation to *O*-glucosides. Nilsson et al. (1996) and Faiss et al. (1996) concluded that *rol*C does not hydrolyze endogenous cytokinin glucosides *in planta*. They proposed that *rol*C expression either leads to a modified synthesis of cytokinins or that the altered cytokinin metabolism is a secondary effect.

The observation that the presence of *rol* genes increases adventitious root formation on a number of mircropropagated cuttings even in the absence of exogenous auxin supports the idea that *rol* genes increase auxin sensitivity. In the apple rootstock M26, Lambert et al. (1998) found that transformation by *A. rhizogenes* led to an increased auxin concentration in leaves and decreased cytokinin and abscisic acid (ABA) levels. The increased auxin concentration is probably due to auxin biosynthesis from the presence of the *aux* genes from the TR-DNA. In 35S-*rol*C and rbcS-*rol*C transgenic aspen, ABA levels were considerably lower and quantification of gibberellin (GA) levels indicates that *rol*C affects GA biosynthesis rather than GA catabolism (Nilsson et al., 1996). Levels of IAA and the metabolites of ethylene biosynthesis were not significantly changed. Martin-Tanguy et al. (1993) noted a

reduction in polyamine and ethylene production during floral development in tobacco transformed with the 35S-*rol*C gene with a double enhancer.

Jasik et al. (1997) studied the effect of various inhibitors of hormone action. The results indicate the role of *rol* genes is complex and several factors probably contribute to the expressed phenotypes as they were unable to mimic effects with inhibitors.

Rol genes clearly interact with several plant hormonal processes and the plant phenotype changes are likely to be due to changes in hormone balance or in hormone signal perception. The observation that similar alterations in growth form can be induced by application of hormones supports the suggestion that *rol* genes act by altering growth regulator metabolism or activity. For example, auxins cause increased rooting, cytokinins result in a bushy appearance, leaf wrinkling and dwarfness can be caused by inhibitors of gibberellin synthesis. The primary effect of the *rol* genes is still to be identified but they do appear to modify plant form through secondary effects on both hormone synthesis and sensitivity.

Altered Phenotype

A range of phenotype alterations, both desirable and undesirable, are caused by transformation with *A. rhizogenes* (reviewed in Christey, 1997). The characteristic altered phenotype induced by *A. rhizogenes* transformation is usually regarded as undesirable, but these altered phenotypic features can have potential applications for plant improvement. For example, in the horticultural industry some morphological alterations such as dwarfing, increased rooting, altered flowering, wrinkled leaves and/or increased branching are desirable.

Pellegrineschi et al. (1994) improved the ornamental quality of a scented *Pelargonium* species by *A. rhizogenes* transformation. This plant has a pleasant odour but is unattractive due to its long internodes and chaotic, ungainly growth. Hairy root regenerants were of shorter stature, with increased leaf and branch production. In addition to improving ornamental quality, other associated benefits included increased rooting of cuttings, altered root system architecture, inhibition of flowering and increased production of essential oils.

A dwarfing response, due to reduced internode distances, is often noted in hairy root regenerants, e.g. *Ajuga reptans* (Tanaka and Matsumoto, 1993), prairie gentian (Handa et al., 1995) and gentian (Suginuma and Akihama, 1995). In snapdragon, flower number was increased dramatically due to increased branching (Handa, 1992). These phenotypes may be of particular benefit for ornamental potted plants.

In addition to alterations in vegetative morphology, plants transformed with *A. rhizogenes* often show alterations in their life cycle. Damiani and Arcioni (1991) noted some perennial forage legumes became annual, while others remained perennial but lost or delayed the capacity to flower. Carrot and chicory, which are normally biennial, became annual when transformed with *A. rhizogenes* (Tepfer, 1984; Sun et al., 1991; Kamada et al., 1992). Other flowering alterations noted include inhibition of flowering, e.g. *Ajuga reptans* (Tanaka and Matsumoto, 1993) and *Pelargonium* (Pellegrineschi et al., 1994), and in gentian accelerated flowering was noted (Suginuma and Akihama, 1995).

In *Gentiana punctata*, Vinterhalter et al. (1999) noted precocious formation of flower buds without the need for vernalization and flowering under *in vitro* conditions. The introduction of flowering without vernalization would be of particular benefit in those crops where vernalization is required for flowering, as it can be difficult to ensure the correct induction time and temperature are maintained. In swede and turnip no alteration in flowering induction has been noted as *A. rhizogenes*-derived plants still require vernalization (Christey, unpublished observations).

Transformation with *Rol* Genes

In addition to the full hairy root phenotype induced by *A. rhizogenes*, it is possible to obtain more specific alterations in morphology and/or development by introduction of specific *rol* genes. This may produce genetic variability useful for incorporation into breeding programs. *Rol* genes have been introduced into a range of plants individually or in combination to assess their expression in plants and to study effect on plant growth and development (Table 2). The full hairy root phenotype is present in transformants expressing all *rol* genes, whereas single *rol* genes cause specific abnormalities. For example, *rol*A causes leaf wrinkling and reduced flowering, *rol*B causes increased auxin sensitivity and an increase in adventitious root formation, and *rol*D can cause dwarfing and early flowering. *Rol*C results in a wide range of alterations in morphology and development including dwarfness, shorter internodes, increased branching, reduced flower size, lack of apical dominance, reduced fertility, reduced chlorophyll content, and altered leaf and stem size. Overall, plants are smaller and more bushy. Table 2 summarizes the 23 different plant species that have been transformed via *A. tumefaciens* with various *rol* genes, in particular *rol*C. While most examples are from the Solanaceae, a diverse range of plants has been studied including horticultural, ornamental and forestry crops. In all cases the most striking alterations are noted in *rol*C transformants.

Even though the exact mode of action of *rol*C is not known it has been widely used for the alteration of plant form and morphology. *Rol*C has been a particularly useful tool for the development of ornamental crops due to the alterations obtained in several commercially important traits. For example, in *Osteospermum ecklonis* (Compositae) plants were produced with early flowering and increased flower number. In addition changes in growth habit, root morphology and leaf color were achieved (Table 2).

These phenotypic effects are generally similar even in taxonomically diverse species, but differ depending on promoter used and copy number and expression effects. Use of the CaMV 35S promoter generally results in more extreme effects, compared to use of the native promoter. For example, northern analyses in *Atropa belladonna* showed that the *rol*C gene driven by the CaMV 35S promoter was strongly expressed in leaves, flowers, stems and roots while the *rol*C gene with its own promoter was expressed only in low levels in these organs (Kurioka et al., 1992). In aspen transgenic for 35S-*rol*C, plants were reduced in size with smaller leaves, whereas rbcS-*rol*C aspen transgenic plants were only slightly reduced in size (Fladung et al., 1997b; Table 2). In tobacco *rol*A retarded flowering under the control of its native promoter, but with the 35S promoter flowering was completely inhibited (Martin-Tanguy et al., 1996).

It is important to determine gene expression levels and correlate this with the extent of phenotype alteration. In petunia, Winefield et al. (1999) characterized in detail five transgenic lines expressing

TABLE 2

EXAMPLES OF PHENOTYPE CHANGES INDUCED BY *ROL* GENE TRANSFORMATION

Plant	Promoter-gene[a]	Phenotype	Reference[b]
Actinidia deliciosa	*rol*ABC	Ri phenotype; increased lateral shoot formation, root system and rooting of cuttings; reduced internode length	Rugini et al., 1997
	*rol*B	Normal phenotype, increased rooting of cuttings	
Apple rootstock M26	*rol*A	Reduced stem growth, leaf area and internode length	Holefors et al., 1998
	*rol*B	Increased rooting, reduced growth	Welander and Zhu, 2000
Atropa belladonna	35S-*rol*C	Increased flowering, reduced apical dominance, pale lanceolate leaves, smaller flowers	Kurioka et al., 1992
	*rol*C	Similar to non-transgenic	
Begonia tuberhybrida	*rol*ABC	Dwarfing, delayed flowering, wrinkled leaves and petals	Kiyokawa et al., 1996
Chrysanthemum	*rol*C	Bushy plants with more dissected leaves	Dolgov et al., 1997
Dianthus caryophyllus	35S-*rol*C	Reduced apical dominance, increased lateral shoot number, increased flowering, increased rooting	Zuker et al., 2001
Lactuca sativa	*rol*AB	More responsive to auxin, dwarfing, increased rooting, leaf wrinkling	Curtis et al., 1996
Lotus corniculatus	*rol*AB; *rol*ABC	Similar to controls	Požárková et al., 1995
	35S-*rol*C	Dwarfing, increased branching, shorter internodes, slender pale leaves, increased root mass	
Lycopersicon esculentum	*rol*A	Severe leaf wrinkling, reduced flower length	van Altvorst et al., 1992
	*rol*B	Reduced apical dominance and internode length	
Medicago sativa	*rol*A; *rol*AB	Similar to controls	Frugis et al., 1995
	*rol*B	Later flowering, increased stem number and root mass	
	*rol*C; *rol*BC	Increased stem number and root mass	
Nicotiana tabacum	*rol*A	Dwarfing, leaf wrinking	Hänisch ten Cate et al., 1988
	*rol*C	Reduced height, earlier flowering, smaller leaves, flowers and seed capsules	Scorza et al., 1994
Osteospermum ecklonis	35S-*rol*C	Increased flower number, early flowering, erect habit, pale green leaves	Giovannini et al., 1999
	*rol*AB	Erect habit with dark green leaves	
	*rol*ABC	Increased number of flowers and branches, early flowering, erect habit	
Pelargonium×*domesticum*	35S-*rol*C	Reduced height, leaf area, petal area and flower diameter	Boase et al., 1998, 1999
Petunia axillaria× (*P. axillaria*×*P. hybrida*)	35S-*rol*C	Reduced height, fertility, leaf and flower size, increased branching, reduced time to flowering	Winefield et al., 1999
Poncirus trifoliata	35S-*rol*C	Dwarfed, reduced internode length, higher rooting	Kaneyoshi and Kobayashi, 1999
	*rol*C	Dwarfed, altered internode length, higher rooting	
Populus tremula	35S-*rol*C	Reduced height, smaller paler green leaves	Fladung et al., 1997a,b
	rbsS-*rol*C	Paler green leaves	
Populus tremula×*P. tremuloides*	35S-*rol*C	Reduced height and internode length, early bud release	Fladung et al., 1996
	rbsS-*rol*C	Smaller paler leaves	
Pyrus communis	*rol*C	Reduced height, number of nodes and leaf area	Bell et al., 1999
Rhaponticum carthamoides	35S-*rol*C	Dwarf phenotype	Orlova et al., 2000
Rosa hybrida	*rol*C	Dwarf phenotype, leaf wrinkling, reduced root system, smaller flowers, numerous thorns	Firoozabady et al., 1994
Rosa hybrida rootstock	*rol*ABC	Reduced apical dominance, decreased shoot length, wrinkled leaves, enhanced rooting on cuttings	van der Salm et al., 1997
Solanum aviculare	35S-*rol*ABC	Reduced growth, suppressed apical dominance, shortened internodes, smaller, sterile flowers	Jasik et al., 1997
S. dulcamara	*rol*AB	Dwarfed, leaf wrinkling, bushy, reduced leaf area, flower size and fertility	Curtis et al., 1999
	35S-*rol*C	As above, reduced chlorophyll and carotenoids	
S. tuberosum	35S-*rol*C	Reduced apical dominance, bushy, smaller paler leaves, fewer smaller tubers	Fladung, 1990

[a] The native promoter was used if no promoter is listed. 35S, cauliflower mosaic virus 35S promoter; rbsS, light-inducible promoter from large subunit of potato ribulose bisphosphate carboxylase.

[b] This is not a comprehensive list, but provides one key reference for each plant.

differing levels of *rol*C transcripts. The following phenotypic alterations were found to increase in severity with increasing *rol*C transcript abundance: reductions in plant height, leaf and flower size, a break in apical dominance leading to increased branching, and decreased male and female fertility. Time to flowering was also reduced in *rol*C transgenic plants. In *rol*C aspen, northern analysis indicated that plants with the most pronounced phenotypic alterations showed the highest levels of *rol*C expression (Nilsson et al., 1996).

In apple rootstock, two clones transgenic for one copy of *rol*B were obtained. Northern analysis showed that the clone with higher *rol*B gene expression had higher rooting ability (Welander et al., 1998; Welander and Zhu, 2000). In aspen, loss of altered leaf shape and wrinkling was correlated with lack of *rol*B and *rol*C

transcripts in older plants (Tzfira et al., 1999). Methylation may account for differences in phenotype. In endive, methylation was noted in plants with an attenuated phenotype (Limami et al., 1998).

An alternative approach to *A. tumefaciens* transformation with *rol* genes is to use *A. rhizogenes* strains with specific *rol* genes knocked out. In *Antirrhinum*, Newbury and Senior (2001) showed inactivation of *rol*A produced plants with less severe Ri phenotype.

Alteration of Root Systems: Increased Rooting

In some species, particularly woody plants, rooting ability is a factor limiting commercial micropropagation. Due to the highly branching root system induced by *A. rhizogenes*, intentional inoculation with *A. rhizogenes* has been used to improve the rooting of cuttings from some recalcitrant crops, particularly woody species. As reviewed in Christey (1997), *A. rhizogenes* is an effective alternative method as improved rooting has been demonstrated in a range of species including *Pinus* sp. and *Larix laricina* (McAfee et al., 1993), *Eucalyptus* species (Macrae and Van Staden, 1993), hazelnut (Bassil et al., 1991) and almond (Damiano et al., 1995). Roy (1989) demonstrated a 10–20% increase in the percentage of rooted cuttings from softwood cuttings of several fruit trees and herbaceous species including peach, apple, cherry, olive, *Choisque ternate*, *Elaeagnus pungens*, *Magnolia soulangeane*, *Pieris japonica*, and *Viburnum tinus*. Rugini and Mariotti (1991) demonstrated successful rooting of olive, apple, almond, and pistachio apical cuttings. Untreated cuttings did not root. Strobel et al. (1988) conducted a field experiment over 41 months that demonstrated the long-term benefits of *A. rhizogenes* inoculation of bare rootstock olive trees.

In apple, jojoba (*Simmondsia chinensis*), walnut (*Juglans regia*), cashew nut (*Anacardium occidentale*), black pine (*Pinus nigra*), jujube (*Ziziphus jujuba*), and coast redwood (*Sequoia sempervirens*), the highest frequency of root induction was obtained when *in vitro* cuttings were inoculated with *A. rhizogenes* (Patena et al., 1988; Caboni et al., 1996; Das et al., 1996; Hatta et al., 1996; Mihaljević et al., 1996, 1999; Benavides and Radice, 1998). In most cases root number was also increased. Dependent on the strain, rooting in jojoba ranged from 73% to 80% compared to 33% on untreated controls. In walnut, 59% rooting was obtained, compared to no rooting in controls. In cashew, even though rooting was improved with *A. rhizogenes*, plant recovery was not improved compared to indole-3-butyric acid (IBA) treatment. In coast redwood roots formed on 58–69% of explants, compared to <20% for controls. In black pine roots formed on 60–97% of cuttings compared to <14% for controls. In both coast redwood and black pine, the roots that formed did not have a hairy root phenotype (Mihaljević et al., 1996, 1999).

Transformation with *rol* genes is another method to exploit the root-inducing effects of *A. rhizogenes*. Nearly all plants listed in Table 2 have shown increased rooting. In orange, almost all *rol*C transformants had rooting abilities greater than the controls (Kaneyoshi and Kobayashi, 1999). The presence of the *rol*ABC genes enhanced root formation in *Rosa hybrida* (van der Salm et al., 1997), kiwifruit (Rugini et al., 1991), and *Solanum aviculare* (Jasik et al., 1997). In addition, explants from transgenic *S. aviculare* plants were able to produce roots without auxin. In several cases an increase in the number of roots per rooted cutting were also obtained, e.g. rose, apple, and kiwifruit.

These examples demonstrate an agricultural advantage to root induction either by *A. rhizogenes* or by specific *rol* gene(s). Such methods have the potential to increase the efficiency of plant propagation in crops where root formation is difficult. In addition, such methods may enable rooting at non-optimal times of the year. Host range analyses are important to conduct as different *A. rhizogenes* strains produce differences in the rooting of different cuttings (e.g. Patena et al., 1988; Roy, 1989; Mihaljević et al., 1996). In addition, further studies on the subsequent development of the cuttings or plants, both in the greenhouse and field, is required. It is important to ascertain that the *A. rhizogenes*-derived root system is fully functional in water-nutrient transport, therefore providing long-term benefits.

Transgenic Rootstocks

Some plants such as fruit trees are often grafted onto a rootstock to alter form and to increase vigor and disease resistance. Dwarfing rootstocks are widely used for reducing tree size, however some commercial rootstocks have features that require improvement, e.g. difficulties in rooting, and non-ideal dwarfing effect. As genetic improvement by traditional breeding is slow, the use of transformation offers many advantages. The introduction of *rol* genes are particularly attractive as the phenotype changes induced, such as increased rooting and dwarfing, are those sought after for rootstocks. Transgenic plants containing various *rol* genes have been produced in kiwifruit, apple, rose, orange, and pear in an effort to alter scion growth habits (Table 2).

In kiwifruit, when *rol*ABC transgenic plants were used as rootstocks grafted with a non-transformed scion, the vegetative habit of the scion was altered with increased lateral branching (Rugini et al., 1997).

The semidwarf apple rootstock requires an increase in rooting ability and is not dwarf enough. However, through transformation with *rol*A and *rol*B separately, a dwarfed phenotype and increased rooting ability were conferred (Holefors et al., 1998; Welander et al., 1998). Subsequent research studied the effect of the transgenic rootstock on growth characters of the cultivar grafted onto it. The results indicate that there was no effect of the transformed rootstock on relative growth rate under non-limiting conditions. However, grafted plants with *rol*A1 rootstock did show a reduced stem, internode and root length, indicating that *rol*A1 might be a potential dwarfing rootstock for apple production (Zhu and Welander, 2000).

In *Rosa hybrida* the presence of *rol*ABC in the rootstock stimulated the formation and initial growth of adventitious roots, and after 3 months the transformed rootstocks were still larger. In addition, the release of basal shoots of the scion were significantly higher on transformed rootstocks than on control rootstocks (van der Salm et al., 1996).

In cherry it would be desirable to have rootstocks with which to modify the vegetative habit of sweet cherry scions. Gutièrrez-Pesce et al. (1998) regenerated plants of cherry rootstock after *A. rhizogenes*-mediated transformation. The plants obtained showed the hairy root phenotype to varying degrees and experiments are in progress to assess the effect of the transgenic rootstock on the growth characteristics of grafted scions. Other plants such as orange (Kaneyoshi and Kobayashi, 1999) and pear (Bell et al., 1999) have

been transformed with *rol*C with the eventual aim to assess their potential as rootstocks for altering scion growth, in particular as dwarfing rootstocks.

Root System Modification

A. rhizogenes-mediated transformation has the potential to introduce foreign genes specifically into the root system, e.g. resistance to root pathogens or pests, or resistance to heavy metals. Hairy root cultures represent an ideal system to study root function, the effects of root pathogens and for testing genes that might cause pathogen resistance. Cho et al. (2000) demonstrated that the soybean cyst nematode could complete its entire life cycle on soybean hairy root cultures. Kifle et al. (1999) have produced sugar beet hairy roots transgenic for nematode resistance and the production of hairy roots provided a system for studying gene function in roots.

An alternative strategy for evaluating the character of transformed roots is the production of composite plants. These are generated by inducing transformed roots on non-transformed shoots. These have been widely used in legumes including *Lotus corniculatus* (Hansen et al., 1989), *Trifolium repens* (Díaz et al., 1989), *Vicia hirsuta* (Quandt et al., 1993), and *Arachis hypogaea* (peanut, Akasaka et al., 1998) as they represent an ideal system for analyses of gene expression involving infection of rhizobia and nitrogen fixation. The advantage of this system is the speed with which a fully developed and nodulated transgenic root system can be obtained. This system is simple and fast, applicable to a range of legumes and is independent of shoot regeneration. In *Vicia hirsuta* nodule development, morphology and function of the transgenic nodules was comparable to the wild type (Quandt et al., 1993). In *Trifolium repens* such a system was used to introduce the pea lectin gene into white clover roots. These roots could then be nodulated by a *Rhizobium* strain usually specific for the pea cross-inoculation group, demonstrating the involvement of lectins in host-plant specificity (Díaz et al., 1989).

This composite plant system could be applicable to a wide range of other studies involving root processes. In grape, hairy root cultures were obtained expressing the coat protein of chrome mosaic nepovirus. Plants were not regenerated but these transgenic roots were successfully grafted onto nontransformed scions, although disease resistance was not evaluated (Torregrosa and Bouquet, 1997).

Introduction of Agronomically Useful Traits

The demonstration that a wide range of crops can be regenerated from *A. rhizogenes* hairy roots (Table 1) is an essential prerequisite to introducing foreign genes for traits of agricultural interest. Of the transgenic plants obtained to date from *A. rhizogenes*-mediated transformation, half have had foreign gene(s), such as reporter and selectable marker genes, introduced in addition to the Ri T-DNA (Table 1). However, there are also examples where Ri-mediated transformation has been used for the introduction of agronomically useful traits. For example, in kidney vetch the *ipt* gene was introduced, and in larch, plants transgenic for *Bt* were obtained. In snapdragon, *Scoparia dulcis*, and *Atropa belladonna* the *bar* gene was introduced. In *Lotus corniculatus* the maize anthocyanin regulatory gene *Sn* was introduced. Some plants were obtained with reduced foliar proanthocyanidin which may be of benefit in some agronomic situations (Damiani et al., 1999).

Ri-mediated transformation for the introduction of agronomically useful traits has been widely used in forage and vegetable brassicas. Chlorsulfuron-resistant turnip, forage rape, and forage kale have been produced (Christey and Sinclair, 1992). Basta-resistant forage rape, forage kale, and swede have been produced and some lines field tested (Christey et al., 1999b, c; Christey and Braun, 2001). In broccoli, plants with reduced ethylene production have been produced and field tested after the introduction of antisense 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase. Plants show reduced ethylene production but no increase in shelf life (Henzi et al., 1999a, b, 2000b). In cauliflower, the magainin and Shiva antibacterial genes have been introduced (Braun et al., 2000).

Woody Plant Improvement

An important goal of tree breeding is altered plant form and performance, especially increased rooting, increased growth and larger wood mass. For this reason woody plant improvement has made increasing use of *A. rhizogenes*-mediated transformation and in particular *rol* gene(s) transformation. Transformation with *rol* genes has been of particular interest due to the phenotypic and hormone changes induced due to the role of hormones, especially auxin, in wood formation.

Research is the most developed in aspen where several years of field testing has been conducted on *rol* transgenic plants. *A. rhizogenes*-mediated transformation and *rol*C gene transformation has been shown by several groups to alter aspen plant growth and development. Tzfira et al. (1998) noted improved root system characteristics, including better rooting ability, improved rooting percentages, and extensive root formation in *A. rhizogenes*-derived aspen. In addition, rooting was more rapid and was not seasonal. In subsequent studies Tzfira et al. (1999) noted several phenotypic changes which were correlated with *rol* gene expression, including breaking of stem apical dominance which resulted in axillary bud break. Transgenic plants also showed accelerated growth in their first year and delayed winter dormancy.

Expression of the *rol*C gene in transgenic aspen has resulted in dwarfness, breaking of apical dominance, reduced growth rate, and decreased internode length (Fladung et al., 1996, 1997a; Nilsson et al., 1996). Grünwald et al. (2000) studied wood formation in 35S-*rol*C transgenic aspen. The transgenics were characterized by a dwarfed phenotype, smaller, wrinkled leaves, reduced growth rate, and earlier bud break. However, wood formation did not immediately follow bud break, but was delayed so that it occurred about the same time as in controls. Quantitatively, wood structure was similar to controls, therefore dwarfism of the transgenics is likely due to a reduction in cell number. Atypical latewood formation was noted and cells lacked secondary walls and normal lignification. Wood discoloration and formation of tyloses were conspicuous in all transgenics.

Marker-free Selection

The use of *A. rhizogenes*-mediated transformation enables the development of transgenic plants via marker-free selection through use of hairy root morphology as the primary indicator of transformation. Due to high rates of co-transformation the genes

of interest on the binary vector are likely to also be present. GUS and GFP reporter genes were used by Puddephat et al. (2000) to select transgenic root cultures, eliminating the need to use antibiotic resistance or herbicide resistance as marker genes. Alternatively, having the genes of interest incorporated in the Ri T-DNA also allows for use of hairy root morphology as the primary indicator of transformation eliminating the need for additional marker genes.

Another approach to producing transgenic plants via marker-free selection is the new plant vector system for repeated transformation known as the multi-auto-transformation (MAT) system developed by Ebinuma et al. (1997). This vector contains the isopentenyl transferase (*ipt*) gene, used as a selectable marker, and either the maize transposable element *Ac* or the *R*/RS site-specific recombination system for removing the *ipt* gene from cells of *ipt*-shooty lines. The phenotypically normal shoots that appear from the *ipt*-shoot lines are characterized by excision of the *ipt* gene. The resulting transgenic plants are marker-free. Use of the *R*/RS system for removing the *ipt* gene is more efficient than *Ac* (Sugita et al., 1999). A *rol* type MAT vector system has also been developed using *rol*A, B, C, and D fragments and used to transform *Antirrhinum majus* (Minlong et al., 2000). Hairy root cultures were selected and transferred to shoot regeneration media. A mix of phenotypically normal and abnormal shoots were produced. From the phenotypically normal shoots, PCR and segregation analyses confirmed the presence of marker-free plants. The use of such MAT vectors could be beneficial in a range of crops, particularly woody species. Marker-free transgenic plants are produced without the need for sexual crossing, thus hastening the production of such plants.

Increasing Rates of *A. Rhizogenes* Transformation

As noted with *A. tumefaciens*-mediated transformation, there is a wide range of transformation frequencies obtained when using *A. rhizogenes*, which varies between species and between cultivars. For example, in vegetable and arable brassicas higher rates of hairy root production are noted with *B. campestris* types compared with *B. oleracea*. However, shoot regeneration rates are higher with *B. oleracea*-derived hairy roots (Christey, unpublished observations). There are two main areas to concentrate on to improve transformation rates: manipulation of genotype, and manipulation of bacteria and transformation conditions. In eight commercial cultivars of broccoli, representing four crop types, Cogan et al. (2000) found that the proportion of inoculated explants with transgenic roots ranged from 1.4% to 58% between cultivars. They identified easy- to- and difficult-to-transform cultivars and produced doubled haploid lines to provide material for investigating the genetic basis of genotypic variation. In two lines from Hawke, transgenic root production increased by over 100%; transformation was also reduced by over 60% in two lines. Transgenic root production was increased by over 400% in three lines of Trixie (Puddephat, personal communication).

In broccoli, Henzi et al. (2000a) studied a range of chemicals including acetosyringone, mannopine and arginine, and factors such as feeder layers, that have been shown to affect transformation rates in other crops. An improved *A. rhizogenes* broccoli transformation system was developed by optimizing several media and factors. Dramatic increases in GUS expression and hairy root production in Shogun broccoli leaf explants were obtained using this new protocol. Puddephat et al. (2001) obtained increased rates of hairy root production in a difficult-to-transform cultivar by inclusion of 2,4-dichlorophenoxyacetic acid (2,4-D) in the medium used to resuspend the *Agrobacterium* prior to inoculation. Puddephat et al. (2000) also found that use of *A. rhizogenes* strain LBA9402 gave higher transformation rates than A4T and also resulted in genotype-independent transformation in broccoli. Kifle et al. (1999) developed an improved transformation protocol for *A. rhizogenes*-mediated transformation of sugar beet by co-inoculation with two strains of *Agrobacterium*. Compared to inoculation with *A. rhizogenes* only, co-inoculation with *A. tumefaciens* and *A. rhizogenes* dramatically increased both the percentage of explants yielding hairy roots and the percentage of GUS-positive hairy roots. In flax, Zhan et al. (1990) noted that hairy root induction was markedly increased by co-inoculation with an *A. tumefaciens* strain containing the cytokinin gene (*tzs*) responsible for *trans*-zeatin synthesis.

In other cases *A. tumefaciens*-mediated transformation has been improved by use of factors such as reducing ethylene accumulation by use of porous tape. It is likely that these variables will be equally applicable to improving rates of *A. rhizogenes*-mediated transformation.

Shoot regeneration from hairy roots often occurs spontaneously (reviewed in Christey, 1997). There are also many plants where shoot regeneration from hairy roots has not been achieved (e.g. mung bean, peanut, and sugar beet), or is very difficult (e.g. *B. campestris*). It is likely that alterations in environmental conditions will enable regeneration from hairy roots. For example, the use of ethylene inhibitors is beneficial for improving shoot regeneration in brassicas and thidiazuron (TDZ) promoted regeneration from *Brassica* hairy root cultures (Christey et al., 1997).

Normal Phenotype

One perceived disadvantage of *A. rhizogenes*-mediated transformation, probably limiting its use for the introduction of foreign genes, is the altered phenotypes often obtained. As discussed above, this altered phenotype can be advantageous in some ornamental and horticultural crops but is usually undesirable in crop plants.

In plants where Ri phenotypic effects are present and undesirable, it is still possible to obtain phenotypically normal transgenic plants. When binary vectors are used, due to the independent insertion of the Ri and Ti T-DNAs, segregation of the T-DNAs at meiosis can occur in subsequent generations. This allows the identification of phenotypically normal transgenic plants, transformed with only the binary vector T-DNA containing the foreign DNA of interest but without the Ri genes and therefore the associated phenotypic changes. The independent segregation of the hairy root phenotype from the other transgenes has been demonstrated in tobacco (Hatamoto et al., 1990), oilseed rape (Boulter et al., 1990), cauliflower (Braun et al., 2000; Puddephat et al., 2001), and broccoli (Puddephat et al., 2001).

Field evaluation of 21 lines of vegetable brassicas from five types indicated a range of phenotypes with four (20%) transgenic lines having a normal phenotype, eight (40%) a moderate and eight (40%) a severe Ri phenotype (Christey et al., 1999a). In addition, one cauliflower line segregated for a normal and severe Ri phenotype. In a field trial of transgenic Basta-resistant forage

kale and forage rape, Ri phenotype effects were obvious in all lines, with four (66%) lines showing moderate or slight Ri phenotype effects and two (33%) lines showing severe Ri effects (Christey and Braun, 2001). PCR analysis indicated that the normal phenotype in broccoli and cauliflower was not strictly correlated with absence of *rol*C as all but one normal cauliflower line still contained the *rol*C gene (Christey et al., 1999a). This demonstrates that phenotype loss can occur by gene silencing in addition to independent segregation of the T-DNAs. This indicates the importance of confirming phenotype by molecular methods to ensure the *rol* genes are absent as gene activation may occur in later generations. A range of Ri phenotypes has been noted in other species including tobacco (Tepfer, 1984), *Lotus corniculatus* (Webb et al., 1990), and potato (Montanelli and Nascari, 1991).

In addition, there are examples where transgenic plants show no or minimal Ri phenotype effects. Manners and Way (1989) obtained some transgenic *Stylosanthes humilis* plants with normal phenotype. Molecular analysis revealed all plants with normal phenotype contained the binary vector T-DNA but one lacked the TL-DNA. In *Nicotiana* sp., Sinkar et al. (1988) noted that after several months growth, normal shoots developed from basal axillary buds of plants with the Ri phenotype. Molecular analysis indicated transcriptional inactivation of TL-DNA in revertant normal shoots. Christey et al. (1994) obtained two lines of forage rape which were barely distinguishable from the non-transgenic control. In transgenic *L. corniculatus*, only a few minor changes in plant morphology were noted, even though two or more copies of TL-DNA were present (Webb et al., 1990). In rapid cycling cabbage, Berthomieu and Jouanin (1992) obtained transgenic roots with no Ri phenotypic characteristics. Some plants were also normal, though with reduced male fertility. Molecular analysis demonstrated the presence of TL- and TR-DNA. There are several possible explanations for these reduced Ri phenotypic effects. In some cases, molecular studies have shown lack of insertion or expression of some or all of the Ri T-DNA. In addition, genotype, copy number and position effects could be responsible for the variation in Ri phenotypic effects in some transgenic plants.

In some cases the direct regeneration of transgenic shoots from explants, with no intervening hairy root phase, has been noted after *A. rhizogenes*-mediated transformation. In Mexican lime, shoots regenerated directly from 76% of explants. Phenotype alterations were noted in some plants (Pérez-Molphe-Balch and Ochoa-Alejo, 1998). In kiwifruit, Yamakawa and Chen (1996) achieved *A. rhizogenes*-mediated transformation by direct formation of adventitious buds on infected petioles. A mixture of normal and Ri phenotype plants were obtained. In broccoli direct regeneration of normal phenotype shoots after *A. rhizogenes*-mediated transformation has also been noted. PCR analysis confirmed the presence of *rol*C (Christey, unpublished observations). In rose, transgenic shoots were obtained after co-cultivation of friable embryogenic tissue with *A. rhizogenes* (Firoozabady et al., 1994).

Conclusions

While the precise mechanism whereby *rol* genes confer the Ri phenotype is not totally known, this has not been a hindrance in exploiting these genes for agricultural and horticultural uses ranging from alteration of plant phenotype and form to introduction of foreign genes. Use of *A. rhizogenes* and *rol* genes has tremendous potential for genetic manipulation of plants and has been of particular benefit for improvement of ornamental and woody plants. In addition, *rol* gene transformation has also provided insights into the function of *rol* genes.

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